Developmental Neuroscience

Dev Neurosci 2002;24:114–124
DOI: 10.1159/000065693

Received: March 21, 2002
Accepted: May 3, 2002

Numerous Proteins in Mammalian Cells Are Prone to Iron-Dependent Oxidation and Proteasomal Degradation

Steven K. Drake[a] Emmanuel Bourdon[a] Nancy B. Wehr[b] Rodney L. Levine[b]
Peter S. Backlund[a] Alfred L. Yergey[a] Tracey A. Rouault[a]

[a]National Institute of Child Health and Human Development, and [b]National Heart Lung and Blood Institute, Bethesda, Md., USA

Key Words
Iron · Oxidation · Carbonyl · Proteasome · Iron regulatory protein 2 (IRP2) · Degradation · Aging · Neurodegeneration · Inclusions · Heat shock proteins

Abstract
The mechanisms that underlie iron toxicity in cells and organisms are poorly understood. Previous studies of regulation of the cytosolic iron sensor, iron-regulatory protein 2 (IRP2), indicate that iron-dependent oxidation triggers ubiquitination and proteasomal degradation of IRP2. To determine if oxidization by iron is involved in degradation of other proteins, we have used a carbonyl assay to identify oxidized proteins in lysates from RD4 cells treated with either an iron source or iron chelator. Protein lysates from iron-loaded or iron-depleted cells were resolved on two-dimensional gels and these iron manipulations were also repeated in the presence of proteasomal inhibitors. Eleven abundant proteins were identified as prone to iron-dependent oxidation and subsequent proteasomal degradation. These proteins included two putative iron-binding proteins, hNFU1 and calreticulin; two proteins involved in metabolism of hydrogen peroxide, peroxiredoxin 2 and superoxide dismutase 1; and several proteins identified in inclusions in neurodegenerative diseases, including HSP27, UCHL1, actin and tropomyosin. Our results indicate that cells can recognize and selectively eliminate iron-dependently oxidized proteins, but unlike IRP2, levels of these proteins do not significantly decrease in iron-treated cells. As iron overload is a feature of many human neurological diseases, further characterization of the process of degradation of iron-dependently oxidized proteins may yield insights into mechanisms of human disease.

Introduction

Iron is a necessary nutrient for virtually all cells and mammalian iron homeostasis is accordingly highly regulated [reviewed in Sheth and Brittenham, 2000; Rouault, 2001, and reviews cited therein]. In tissues such as mammalian liver and basal ganglia of the brain, iron content is characteristically high relative to other tissues [Dexter et al., 1992]. In genetic hemochromatosis, abnormal hepatocyte iron content is recognized as a cause of cellular dysfunction and cirrhosis [Brissot et al., 2000]. Increased neuronal iron content may also contribute to pathogenesis in some human neurodegenerative diseases such as Parkinson's and Alzheimer's diseases [Sayre et al., 1999]. Iron is an important bound cofactor of numerous proteins because of its ability to facilitate chemical reactions that

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Dr. Tracey A. Rouault
9000 Rockville, Pike
Bldg. 18 Rm. 101
Bethesda, MD 20892 (USA)
Tel. +1 301 496 6368, Fax +1 301 402 3428, E-Mail trou@helix.nih.gov

involve single electron transfers. In addition, other proteins contain non-iron cation binding sites or accessible cysteines that can dynamically exchange and bind iron if free iron concentrations are sufficiently high. Both specific iron-binding proteins and those that may inadvertently bind iron are at risk from oxidations that occur when protein-bound iron catalyzes the formation of hydroxyl radicals [Stadtman and Oliver, 1991]. Hydroxyl radicals generated by protein-bound iron are highly reactive and often damage residues close to the iron binding site. As a consequence, proteins that undergo iron-dependent oxidation often lose function through loss of amino acid side chains or through peptide backbone cleavage [Stadtman and Oliver, 1991]. Thus, there are many incentives for cells to protect their proteins from exposure to excess free iron.

Regulation of cellular iron metabolism has been extensively studied and iron uptake and sequestration are highly regulated to optimize intracellular iron concentrations [reviewed in Rouault, 2001]. Iron regulatory proteins (IRP1 and IRP2) are cytosolic proteins that post-transcriptionally regulate expression of several iron metabolism proteins, including the transferrin receptor and ferritin, by binding to RNA stem-loops known as iron-responsive elements within the transcripts that encode these proteins. The importance of regulation of iron metabolism is illustrated by the fate of mice in which IRP2 is genetically ablated. Even though these IRP2–/– mice are normal in young adulthood, they develop a late-onset progressive neurodegenerative disease characterized pathologically by axonal iron overload and degeneration, and clinically by tremor and gait abnormalities [LaVaute et al., 2001].

An area of study that has received considerably less attention than regulation of genes involved in iron metabolism relates to whether cells selectively identify and remove oxidatively damaged proteins from their various compartments. The extent to which selective degradation contributes to characteristic turnover rates of individual proteins is often not known. Since mammalian cells have the capacity to efficiently and specifically degrade proteins in proteasomes and lysosomes, it is likely that cells can specifically identify and degrade oxidatively damaged proteins and that the importance of such processes in the response of cells to oxidative stress is not yet fully appreciated.

Over the past several years, we have characterized the iron-dependent degradation of the cytosolic iron sensor, iron regulatory protein 2 (IRP2). In iron-replete cells, IRP2 generally cannot be detected because it is efficiently degraded by the proteasome [Iwai et al., 1995; Guo et al., 1995]. Use of efficient proteasome inhibitors enabled us to analyze the steps that lead to iron-dependent degradation of IRP2 [Iwai et al., 1998]. We determined that purified IRP2 exposed to iron and oxygen in vitro acquired characteristic iron-dependent oxidation and cleavage reactions. In cells treated with proteasome inhibitors, a characteristic iron-dependent oxidation was identified through use of reagents that detect carbonyl formation [Levine et al., 1994]. Use of proteasomal inhibitors also demonstrated that IRP2 was ubiquitinated prior to its degradation and in vitro ubiquitination assays indicated that iron-dependently oxidized IRP2 was the substrate for the ubiquitination reaction. IRP2 contains a degradation domain consisting of 73 amino acids that contains five cysteines and numerous acidic residues, and this domain appears to have evolved into an excellent binding site for free or heme iron. Thus, IRP2 is an effective iron sensor because it undergoes efficient iron-dependent oxidation and proteasomal degradation in iron-replete cells. This mechanism of sensing is both direct and reliable, since the fully degraded protein cannot be inadvertently reactivated [Iwai et al., 1998].

To evaluate whether other proteins in addition to IRP2 are subject to iron-dependent oxidation and degradation, we performed analyses of whole cell lysates from cells grown under a variety of conditions. RD4 cells, a rhabdomyosarcoma cell line, were treated with an iron source or an iron chelator during growth [DeRusso et al., 1995] and lysates were analyzed on two-dimensional gels, separating by both charge and size. A carbonyl assay was performed to identify oxidative modifications [Levine et al., 1994]. In addition, we repeated the same experiment in the presence of proteasomal inhibitors [Fenteany et al., 1995] as we recognized from previous studies of IRP2 that the oxidatively damaged form of IRP2 is absent in living cells that contain functional proteasomes.

Our studies have led to identification of numerous proteins that contain carbonyl modifications in cells treated with both iron and proteasomal inhibitors, but not in cells treated only with iron or with desferal. In this group of proteins, the carbonyl modifications are most readily detected when proteasomal function is inhibited, implying that cells possess mechanisms that allow them to selectively remove oxidized proteins. In the case of IRP2, the process of iron-dependent oxidation and proteasomal removal is efficient and IRP2 levels change markedly depending on intracellular iron concentrations, thus enabling IRP2 to function directly as an iron sensor [Iwai et al., 1998]. In the other proteins we have thus far identified, the oxidatively modified proteins appear to repre-

sent a small fraction of the total protein that is nevertheless efficiently removed in the intact cell. Thus, these experiments imply that there is an active and efficient quality control system in cells that allows for the selective removal of iron-dependently oxidized proteins by the proteasome.

Methods

Cell Growth and Sample Preparation

RD4 cells were grown in DMEM supplemented with 10% fetal bovine serum, 200 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 m*M* glutamine to a density of 50% confluency before being treated for 16 h with either deferoxamine mesylate (Df) (100 μ*M*) or ferric ammonium citrate (FAC) (100 mg/ml) in either the presence or absence of 5 μ*M* lactacystin (Corey Lab, Harvard). These iron treatments do not significantly change cell viability or protein synthesis [DeRusso et al., 1995]. Cells were then washed twice with cold PBS and scraped from the plate. Cells treated with FAC or Df were lysed with 200 μl reaction buffer (8 *M* urea, 0.06% SDS, 3.2% Triton X-100, 120 m*M* DTT, 100 m*M* Df, 40 m*M* Tris, pH 8.0). To this was added 200 μl of 20 m*M* 2-4 dinitrophenylhydrazine (DNPH) (Aldrich D19-930-3) in 10% TFA. After 10 min, the labeling reaction was quenched with 160 μl of 2 *M* Tris base and placed in a Slide-a-Lyser cassette for 45 min vs. 1 liter of 1/20th dilution of reaction buffer.

2D Electrophoresis

The sample was then lyophilized to 100 μl (~6× concentration) and diluted with 150 μl ID sample buffer (5 *M* urea, 4% Triton X-100, 5.5% ampholytes 3–10). Protein assays were done using the Bradford Assay (Pierce). Proteins were separated by 2D electrophoresis according to the manufacturer's instructions (Genomic Solutions, Chelmsford, Mass., USA). Briefly, samples (600 or 2,000 μg) were subjected to IEF separation on a 3 × 260 mm tube gel of 4.4% acrylamide for 18 h using 10 m*M* phosphoric acid and 100 m*M* NaOH as the buffers. The tubes were prepared for the second dimension by extruding them into 50 ml of equilibration buffer (6 *M* urea, 130 m*M* DTT, 30% glycerol, 45 m*M* Tris, 1.6% SDS, 0.002% bromophenyl blue, pH 7.0). The buffer was changed twice at 15-min intervals. They were then laid on a 10% SDS-PAGE gel (1 mm, Genomic Solutions) and overlaid with 1% agarose to cement them in place. These were run until the bromophenyl blue was within 1 cm of the bottom of the gel (~21 cm gel separation, ~5 h). Gels were transferred via the semidry method (Genomic Solutions) for 45 min to either Immobilon-P or nitrocellulose as needed. Carbonyl assays were performed as described in Levine et al. [1995] and gels run in parallel were stained with Coomassie brilliant blue.

Protein and Western Blot Analysis

Proteins transferred to Immobilon-P were visualized by Coomassie staining. To detect carbonyl modifications, nitrocellulose protein blots were probed with anti-dinitrophenyl (DNP) antibodies using described blotting procedures [Levine et al., 2000]. Post-transfer staining of the blots was performed with Amido black according to the same procedures to assess transfer efficiency.

Alternatively, lysates of treated cells were evaluated by SDS-PAGE and transferred to Immobilon-P membranes. ECL blot analysis was performed using antibodies to the following proteins – IRP2 (Rouault Lab, NIH), Cu/Zn superoxide dismutase (RDI), heat shock protein 27 (Stressgen), tropomyosin (Sigma), B23 nucleophosmin (Santa Cruz), actin (Santa Cruz), calreticulin (Novus), heat shock protein 60 (Santa Cruz), and stathmin (Santa Cruz).

Protein Identification

10 mg of RD4 sample was generated as described and run on 5 parallel gels. Gels were stained with Coomassie and images generated from stained gels were overlaid with the results of the anti-DNPH analysis. Colocalizing spots were excised from the 5 gels and submitted separately for identification.

In gel digestion of the SDS-PAGE separated proteins was carried out using a procedure similar to that published by the Association of Biomolecular Resource Facilities (ABRF) [Association of Biomolecular Resource Facilities, http: www.abrf.org ABRF Research Committees intprotseqrescomm.html, 1997]. The excised protein bands were washed and in gel digested using modified porcine trypsin (Promega). The resulting peptides were extracted in two steps, partially dried, redissolved in 0.1% TFA (Aldrich); salts were removed using C18-ZipTip® (Millipore) solid-phase extractions into 10 μl of 1:1 0.1% TFA:acetonitrile.

Two approaches for mass spectrometric analysis were routinely taken for each digest sample. First, 10% of the digest was used for peptide mass fingerprinting by MALDI-TOF mass spectrometry. That is, 1 μl of the extracted digest was applied to a MALDI sample plate along with an equal volume of matrix solution. The matrix was a saturated solution of α-cyanocinnamic acid (Aldrich) in 1:1–0.1% TFA:acetonitrile. A Voyager DE-STR (Applied Biosystems, Framingham, Mass., USA) operated in the reflector mode was used for these analyses. The spectra were used to produce mass lists of ions having signal-to-noise ratios more than 3-fold greater than background. These mass lists were used as input for searching non-redundant mammalian data bases using both ProteinProspector [Clauser et al., 1995] and Mascot [Perkins et al., 1999].

The second approach was to take some portion of the remainder of the extracted peptides and subject them to analysis by LC-MS/MS. The extracts were partially dried and resuspended in 9 μl of 0.1% TFA. An aliquot of this resuspended sample, 2–5 μl, was injected onto a packed capillary gradient MAGIC LC system (Michrom Bioresources, Auburn, Calif., USA) operated at 400 nl/min using a Michrom Magic constant pressure splitter. Samples were separated using a 10-min linear gradient, 2–85% B (A: 5% acetonitrile in water with 0.5% acetic acid and 0.005% TFA; B: 80% acetonitrile in water with 0.5% acetic acid, 0.005% TFA) with a Vydac C18, 5 mm particle, 300 Å pore packing material. Columns, approximately 5 cm in length, were packed by hand using a pressure bomb into a 75-μm ID fused silica capillary PicoFrit® (New Objective, Woburn, Mass., USA). The LC effluent was electrosprayed directly into the sampling orifice of an LCQ DECA (Thermo Finnigan, San Jose, Calif., USA) using an adaptation of the microscale electrospray interface developed by Lee and co-workers [Davis et al., 1995]. The LCQ DECA was operated in a mode that automatically generated MS-MS spectra of the four most intense peaks present in any single scan of the ion tran which exceeded a pre-set threshold. Peptide partial internal sequence data produced by the LC-MS/MS experiments were subjected to analysis using both SEQUEST [Yates et al., 1995] and MASCOT programs employing the mammalian portions of nonredundant data bases.

Table 1. Proteins identified as iron-dependently oxidized and degraded by the proteasome

Spot	Protein identification[1]	Accession No.	Peptides matched	Coverage[2]
1	Cu/Zn superoxide dismutase	4507149	9	127/154 (82%)
2	Peroxiredoxin 2	2507169	10	81/198 (41%)
3	Ubiquitin C-terminal hydrolase	136681	14	141/223 (63%)
4	HSP27	123571	5	50/199 (25%)
5	DJ-1	2460318	6	43/189 (23%)
6	HIRIP5/hNFU1	7661720	5	52/196 (27%)
7	Tropomyosin	37424	36	206/248 (83%)
8	B23 nucleophosmin	825671	13	124/294 (42%)
9	Actin			
	gamma	4501885	18	157/375 (42%)
	beta	4501887	18	157/375 (42%)
10	Calreticulin	4757500	15	179/417 (43%)
11	HSP60	129379	18	284/573 (50%)
12[3]	Calumenin	2809324	13	53/315 (17%)
13[3]	Stathmin	134973	18	92/149 (62%)

Proteins identified by mass spectrometry are listed according to the numbering shown in figure 2. Accession numbers and number of amino acids sequences vs. total amino acid content is shown. For actin, the sequences of gamma and beta are close to one another, and sequencing did not permit a distinction to be made.

[1] Proteins identification was determined from searching the human subset of the non-redundant NCBI database with peptide masses from the MALDI spectra using Mascot. The protein with the highest score for each spot is shown, and in all cases the probability-based Mowse score for the match was well above the level of significance ($p < 0.05$). For proteins 5 and 6, identification was based on database matching for the LC/MS/MS spectra, using Mascot and the merged DTA files generated from the MS/MS spectra using Sequest.

[2] Coverage was calculated from the number of amino acid residues in the matched peptides, divided by the total number of residues in the protein.

[3] Non-oxidized control proteins were numbers 12 and 13, calumenin and stathmin.

In general, the MALDI-TOF approach, while rapid, did not yield convincing identifications of most proteins in these samples. The most reliable results for the identification of the isolated proteins were obtained using the MASCOT probability based searching approach with the LC-MS/MS data.

Results

In cells treated with iron, numerous individual proteins contained carbonyl modifications when compared with cells treated with Df (fig. 1A vs. 1C). Addition of the proteasomal inhibitor lactacystin to iron-depleted cells resulted in a small increase in the number of oxidatively modified proteins within the lysate (fig. 1B). In contrast, addition of lactacystin to cells treated with iron prior to lysis dramatically increased the number of oxidatively modified proteins detected (fig. 1C vs. 1D). Comparisons of Coomassie-stained blots from the four conditions demonstrated that total amounts of distinct and abundant proteins changed little in each of the four treatment conditions.

We selected 11 abundant oxidized proteins that occupied distinctive locations in the gel for identification by mass spectrometry. In figure 2, a representative Coomassie-stained gel is shown, and carbonyl-modified proteins that co-localized with Coomassie-stained proteins are numbered (1–11) both in the carbonyl blot (L panel) and in the corresponding Coomassie-stained gel (R panel). In addition, two additional protein spots (12 and 13) were selected for identification specifically as controls because they could be identified in the Coomassie-stained gel but were not prominently carbonyl-modified. Each of the protein spots was excised from the gel and subjected to MS/MS identification. In table 1, the proteins identified as subject to iron-dependent oxidation are listed and the non-oxidized control proteins detected only on Coomassie staining are indicated.

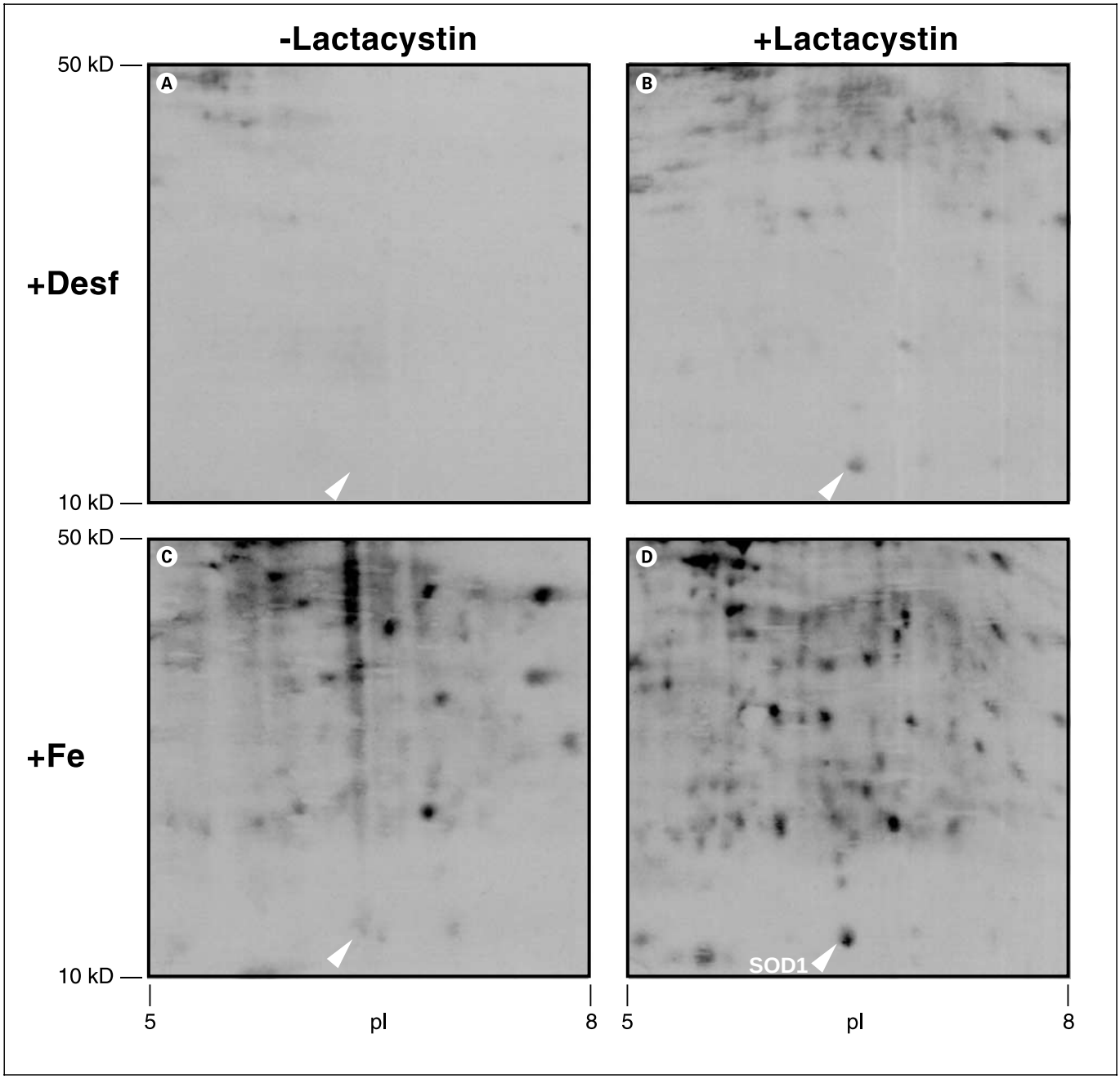

Fig. 1. Many proteins are carbonylated in vivo in the presence of iron and lactacystin. RD4 cells were cultured for 16 h in the presence of either 100 μg/ml ferric ammonium citrate (FAC) (Sigma) or 100 μ*M* deferoxamine mesylate (Df) (Sigma) in the presence or absence of 5 μ*M* lactacystin. Cells were harvested and lysed in a buffered urea solution and derivatized with DNPH (see 'Materials and Methods'). Duplicate samples (600 μg total protein/gel) were separated on preparative 2D gels (Genomic Solution, Chelmsford, Mass., USA) and transferred to membranes for either anti-DNPH blots (shown above) or Coomassie brilliant blue staining (data not shown). Blots shown represent proteins of approximately 10–50 kD and pI 5–8 (left to right). The gels shown are representative of an experiment that was repeated with similar results four times. For purposes of orientation, the spot that corresponds to SOD1 is marked with an arrowhead in all four gels.

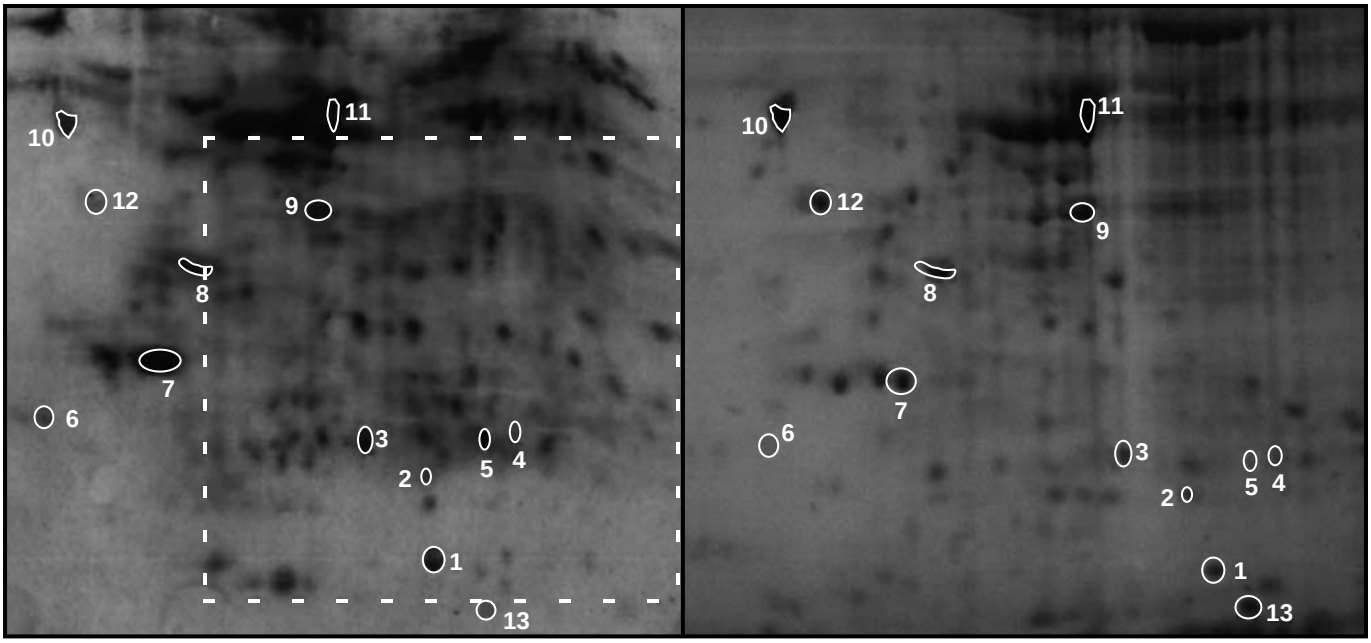

Fig. 2. Oxidized proteins selected for MS identification. Scans of both the iron and lactacystin carbonyl blot and the Coomassie blots were overlaid using the program 2D-Analyzer (Bioimage, Ann Arbor, Mich., USA) running on a Sparc5 Sun system. Only those spots that were overlaid by both the computer and by eye were excised from gels for identification. Spot 1 represents SOD1 (see fig. 1). The remaining spots were as follows: spot 2 = peroxiredoxin 2; spot 3 = ubiquitin C-terminal hydrolase 1; spot 4 = HSP27; spot 5 = DJ-1; spot 6 = HIRIP5/hNFU1; spot 7 = tropomyosin; spot 8 = B23 nucleophosmin; spot 9 = actin; spot 10 = calreticulin; spot 11 = HSP60; spot 12 = calumenin; spot 13 = stathmin. Spots 12 and 13 were selected because they were abundant but were not notably oxidized. Dashed lines indicate area of gel shown in figure 1D.

The most dramatically iron-dependently oxidized protein was cytosolic superoxide dismutase (SOD1, fig. 1, and spot 1, fig. 2). Although carbonyl content changed dramatically depending on iron status, this spot appeared to be equally prominent in Coomassie-stained blots corresponding to all four different treatments of figure 1 (data not shown), and Western blots verified that total levels of SOD1 did not change according to whether the cells were treated with iron (fig. 3). Thus, unlike IRP2 (fig. 3, top), there is no striking decrease in SOD1 total protein in lysates from cells treated with iron, but similar to IRP2, carbonyl content increases in the presence of iron and lactacystin. Notably, when proteasomal function is intact, much less oxidized SOD1 is visible, implying that the oxidized form of SOD1 has been specifically degraded. Thus, either the fraction of this protein that undergoes iron-dependent oxidation and proteasomal degradation over the 16-hour treatment period is small relative to total cellular SOD1, or the cells compensate for the loss of SOD1 that results from degradation.

The iron-dependent oxidation of spots 2 and 5, identified respectively as peroxiredoxin 2 and DJ-1, may also be attributable to metal-catalyzed oxidation. Peroxiredoxin 2, formerly known as thioredoxin peroxidase [Chae et al., 1999; Rhee et al., 1999], is a member of a large family of hydroperoxide reductases that could be vulnerable to damage by high iron when the substrate hydrogen peroxide is bound. DJ-1 has been previously shown to be oxidized in paraquat-treated cells [Mitsumoto et al., 2001].

The gene that encodes the protein at spot 3 is ubiquitin carboxyl-terminal esterase L1 (UCHL1). Spot 3 is one of the most noteworthy spots on the gel, because it is prominently oxidized in cells treated with iron and lactacystin, whereas it is not even perceptibly oxidized in cells treated with Df. Notably, the amount of oxidized UCHL1 is increased when proteasomes are inhibited, implying that,

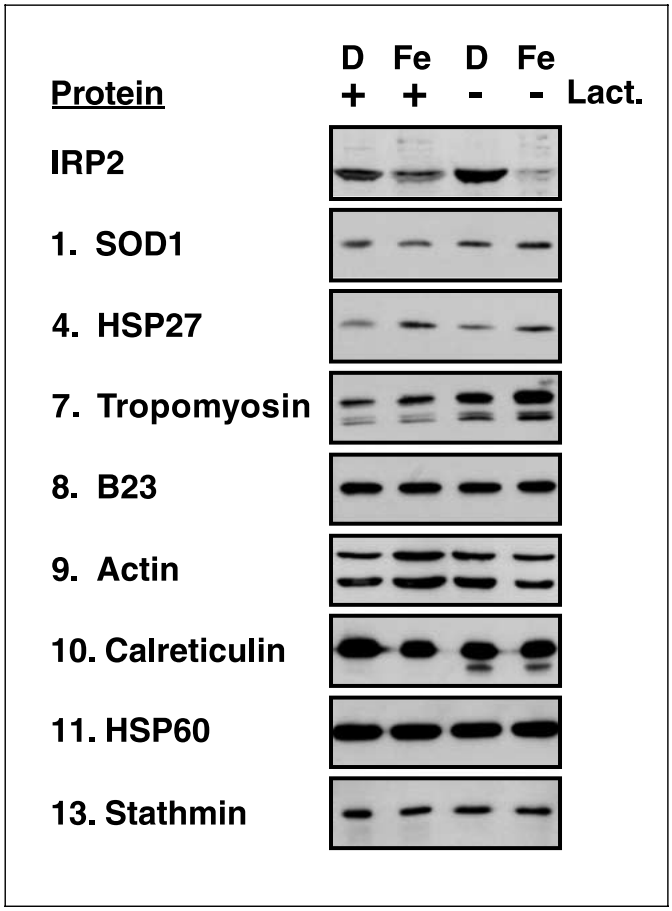

Fig. 3. Selective removal of iron-dependently oxidized proteins does not change total amounts of each protein in iron-treated cells. Lysates of treated cells were separated by SDS-PAGE and transferred to Immobilon-P membranes. Amounts of total protein loaded varied from 2 to 100 μg/lane depending on the antibody being used. Antibodies were used at a dilution of 1:1,000 and visualized using the appropriate HRP-conjugated secondary antibody and the ECL system (Amersham).

like most of the other proteins identified, oxidatively damaged UCHL1 is selectively removed in cells that are exposed to high iron.

B23 nucleophosmin (spot 8) is a mainly nucleolar multifunctional protein [Hingorani et al., 2000] believed to play a role in ribosomal biogenesis, and to function as an ER-specific molecular chaperone [Szebeni and Olson, 1999] in prevention of stress-induced aggregation and restoration of activity of denaturated enzymes.

Although actin is not known to interact with hydrogen peroxide or iron, it is one of the proteins (spot 9) that is prominently oxidized in iron-treated cells. Tropomyosin 4 (spot 7) is one of several myofilament proteins known to be damaged during ischemia-reperfusion [Van et al., 1998]. Despite an obvious increase in oxidative damage, the protein levels of tropomyosin appear to decrease in the presence of lactacystin (fig. 3, lane 7).

Two members of the heat shock family of proteins, HSP27 (spot 4) and HSP60 (spot 11) were unexpectedly identified as being subject to iron-dependent oxidation. HSP-60 is a mitochondrial chaperone that does not have a recognized tendency to iron-dependent oxidation. Although these two proteins are often induced under stress conditions [Feder and Hofmann, 1999; Hartl, 1995], the iron treatments in our experiment induced only a mild increase in HSP27 (fig. 3, #4) and no discernible increase in HSP60 (fig. 3, #11). Visual inspection of these proteins does not reveal obvious potential iron-binding sites.

Calreticulin (spot 10) is a protein with multiple ascribed functions [Park et al., 2001], including function as an ER chaperone, modulation of nuclear-hormone receptor-mediated gene transcription [Holaska et al., 2001] and transport of cytosolic iron [Nunez et al., 2001]. Total levels of calreticulin did not change significantly depending on iron or lactacystin treatment (fig. 3, lane 10).

The most acidic of the iron-dependently oxidized proteins selected for analysis was identified as HIRIP5, a name this protein received upon its identification in yeast as an interaction partner of a histone core binding protein [Lorain et al., 2001]. It is closely related to yeast NFU1 [Schilke et al., 1999], a gene that encodes a peptide that corresponds to the C-terminus of an iron-sulfur cluster assembly enzyme, bacterial NifU [Agar et al., 2000]. NFU1 encodes a modular unit that corresponds to the region of NifU that assembles a [2Fe-2S] cluster [Agar et al., 2000]. Thus, by analogy to bacteria, human HIRIP5 is the human form of NFU1 and is by inference an iron-sulfur protein.

Two spots on the Coomassie gel, spots 12 and 13, were chosen for identification despite the fact that there was little evidence of iron-dependent oxidation. Stathmin (pI = approximately 6.2) [Zugaro et al., 1998] is a microtubule destabilizing protein [Cassimeris, 2002] and calumenin (pI = 4.47) is a member of the EF-hand protein family [Honore and Vorum, 2000]. Both proteins were abundant, and literature reviews and inspection of the protein sequences were consistent with the result that these proteins are not notably oxidized by increases in intracellular iron.

Discussion

In this study, we have identified a group of proteins that are particularly prone to iron-dependent oxidation and proteasomal degradation. In the design of our experiment, the stimulus for oxidation was provided by increased intracellular iron. In addition, inhibition of the proteasome allowed detection of significant increases in oxidative modifications relative to levels detected in cells with functional proteasomes. Previously, several studies have identified proteins that undergo oxidative modification in response to a variety of oxidative stresses. These include oxidation of specific yeast proteins upon treatment with hydrogen peroxide or menadione [Cabiscol et al., 2000], oxidation of hepatic cellular proteins upon hypoxia-reoxygenation of the liver [Reinheckel et al., 2000], and oxidation of human endothelial cell proteins upon exposure to sublethal doses of paraquat [Mitsumoto et al., 2001]. In addition, iron treatment of rat brain lysates leads to oxidation of proteins and lipids, and also to an increase in ubiquitin modified lysate proteins [Adamo et al., 1999]. None of these studies compared levels of oxidized proteins in cells treated with proteasome inhibitors to cells in which proteasomal function was intact.

Using two-dimensional gel technology and mass spectrometry, we have identified 11 proteins that acquire carbonyl modifications in cells treated with iron and proteasomal inhibitors. These proteins were selected for mass spectrometric identification because they were clearly iron-dependently oxidized and were both abundant and distinctively positioned in two-dimensional gels. The proteins identified fall into several distinct groups, and in most instances, a reason for iron-dependent oxidation can be readily discerned.

Two of the proteins identified, calreticulin and hNFU, are proposed to be iron-binding proteins under some circumstances and could therefore be subject to metal-catalyzed oxidation reactions [Stadtman and Oliver, 1991]. Calreticulin may be a cytosolic iron transporter [Nunez et al., 2001]. It has a calculated pI of 4.29 and a highly acidic C-terminus with the sequence 'eeeedkkrkeeeeaedkeddedkdedeedeedkeedeeed', a region that is believed to bind calcium in the ER, but which may also dynamically exchange iron in the cytosol. NFU has been shown to bind an iron-sulfur cluster in bacteria, and as a presumed iron-binding protein, it is not surprising that this protein would be subject to iron-dependent oxidation.

Two of the other iron-dependently oxidized proteins, peroxiredoxin 2 and SOD1, are directly involved with metabolism of hydrogen peroxide, and a third, DJ-1, has been previously been identified as being paraquat-sensitive. Increased intracellular iron may contribute to increased SOD1 and peroxiredoxin 2 oxidation by multiple means. Iron may increase the production of superoxide by 'autooxidation' of cellular metabolites such as NAD(P)H. The dismutation of superoxide by SOD1 may then generate hydrogen peroxide, which may increase the production of more reactive oxidizing species in a site-specific fashion through Fenton chemistry [Koppenol, 1993] or by other chemical means [Yim et al., 1990; Mao et al., 1993]. Although the protein identified as DJ-1 does not have obvious sequence features or conserved functional domains that offer insight into why it is a good candidate for iron-dependent oxidation, it is prone to oxidation in the presence of paraquat [Mitsumoto et al., 2001], as is the aforementioned peroxiredoxin 2. DJ-1 interacts with the androgen receptor [Takahashi et al., 2001] and is proposed as a useful indicator of in vivo oxidative stress [Mitsumoto and Nakagawa, 2001]. Interestingly, DJ-1 is the only protein identified in which carbonyl content does not increase significantly in iron-treated cells when proteasomal function is inhibited, implying that iron-dependently oxidized DJ-1 is not specifically degraded by proteasomes.

Tropomyosin, B23 nucleophosmin, and UCHL1 contain highly acidic stretches that may predispose them to transiently interact with iron and undergo iron-dependent oxidation. B23 nucleophosmin contains two highly acidic amino acid sequences, including the sequence 'deddddddeedddeddddddd' (residues 147–166), that may be important in its chaperone activity. These regions of high acidity could represent electrostatically favorable iron-binding sites that render B23 vulnerable to iron-dependent oxidation. Tropomyosin has not previously been demonstrated to be subject to iron-dependent carbonyl formation, but its multiple stretches of acidic residues (pI calculated = 4.8) are consistent with a possibility for iron-binding and oxidation.

The discovery that UCHL1 undergoes iron-dependent oxidation is relevant to neuroscience because neurons are the primary site of expression of UCHL1, which accounts for up to 2% of total brain protein [Wilkinson et al., 1989], and our data indicate that UCHL1 is also an abundant protein in the muscle derived RD4 cell line. UCHL1 is a member of a family of cysteine proteases whose products hydrolyze small C-terminal adducts of ubiquitin to generate the ubiquitin monomer. The crystal structure of the highly related protein UCHL3 contains an active site catalytic triad of Cys95, His169, and Asp184, and the

molecular surface consists almost entirely of negative electrostatic potential attributable to specific and conserved glutamic acids and aspartic acids, the likely contact points for binding of basic residues in ubiquitin (K6, K11, R72, R74) [Johnston et al., 1997; Wilkinson et al., 1999]. A mutation in UCHL1, Ile93Met, causes partial loss of thiol protease activity and is associated with autosomal dominant Parkinson's disease in a single German pedigree [Leroy et al., 1998]. UCHL1 has also been identified as a constituent of Lewy bodies in diffuse Lewy body disease [Lowe et al., 1990] and is present in axons [Bizzi et al., 1991]. Mice with gracile axonal dystrophy contain an intragenic UCHL1 deletion [Saigoh et al., 1999] and simultaneous genetic ablation of Uch-L1 and Uch-L3 in mice leads to severe axonal degeneration of specific neuronal tracts, including the gracile tract, the nucleus tractus solitarius and the area postrema of the medulla [Kurihara et al., 2001], Thus, the discovery that UCHL1 is prone to iron-dependent oxidative modification may have interesting implications in neurodegenerative diseases.

Actin, HSP27 and HSP60 are not known to interact with iron or hydrogen peroxide. Interestingly, carbonyl-modified actin is significantly increased in the brains of Alzheimer's patients [Aksenov et al., 2001] and is increased in myocardial reperfusion injury [Powell et al., 2001], although in each of these cases, the initial cause of oxidation has not been identified. Functional impairment of actin can result from oxidation [Dalle-Donne et al., 2001]. As increased cellular iron has been reported in Alzheimer's disease and myocardial reperfusion injury [McCord, 1998; Coudray et al., 1994; White et al., 2000], it is possible that iron-dependent oxidation of a subset of vulnerable proteins is an important factor in pathogenesis of these diseases.

Interestingly, HSP27 has previously been detected in inclusions in brains of aging rhesus monkeys, within spheroids (axonal swellings) that also contain ubiquitin, alpha B-crystallin, and ferric iron [Schultz et al., 2001]. HSP27 has not previously been described in the literature as a target of iron-dependent oxidation, but treatment of human endothelial cells with hydrogen peroxide resulted in enhanced expression and increased phosphorylation of HSP27, along with four members of the peroxiredoxin family, including the one identified in this study as peroxiredoxin 2 [Mitsumoto et al., 2001].

Although all of these proteins are iron-dependently oxidized, the percentage of each protein that is oxidized and degraded by the proteasome is sufficiently small during the treatment period that the abundance of each protein does not significantly decrease in response to iron treatment. It is likely that the selective removal of oxidized forms of the protein generally serves a protective role for iron-loaded cells. We have identified 11 iron-dependently oxidized proteins, but many less abundant proteins that are not yet identified also appear to undergo iron-dependent oxidation and proteasomal degradation (see fig. 1).

The effects of iron-dependent oxidation are not necessarily limited to carbonyl formation. Actual cross-linking of proteins may be another effect of iron-dependent oxidation that could have important consequences for cells, since cross-linked proteins may be resistant to unfolding and degradation by the proteasome [Grant et al., 1993; Friguet et al., 1994]. Such proteins could occupy proteasomal binding sites, but resist degradation and thereby disable proteasomal function [Bence et al., 2001]. It is interesting that both UCHL1 and HSP27 have been identified in neuronal inclusions, and actin and tropomyosin have also been found in a particular type of inclusion in Alzheimer's patients known as Hirano bodies [Galloway et al., 1987].

Our results suggest a mechanism by which certain proteins could accumulate in inclusions in patients with neurodegenerative diseases. Aggregation appears to be a fairly specific phenomenon [Rajan et al., 2001] in which abnormally folded proteins do not indiscriminately recruit other proteins. However, accumulation of numerous apparently unrelated proteins into inclusions could be related to a common preceding toxic event that affects a subset of cellular proteins. Iron overload has been described in many disease states including Alzheimer's disease [Sayre et al., 1999], Parkinson's disease [Gerlach et al., 1994]. NBIA1 (neurodegeneration with brain iron accumulation type 1, formerly known as Hallervorden-Spatz disease) [Swaiman, 2001], aceruloplasminemia [Gitlin, 1998] and in IRP2 knockout mice [LaVaute et al., 2001]. Inclusions are a feature of many of these diseases. We propose that iron-dependent oxidation of a subset of proteins that are particularly prone to iron-dependent damage may be an unrecognized early event that leads to inclusion formation when proteasomes are unable to rid the cell of damaged proteins. We also predict that cells will contain ubiquitin ligases that specifically recognize iron-dependent oxidation events and tag modified proteins for degradation. Further work will be needed to elucidate the role of iron-dependent oxidation in settings such as aging, neurodegeneration, and ischemic injury. However, our initial attempt to identify a class of proteins that are particularly subject to iron-dependent oxidation has revealed interest-

ing links to proteins involved in hydrogen peroxide metabolism, to the heat shock response, and to a set of proteins that are commonly found in inclusions in neurodegenerative diseases.

Acknowledgements

This work was supported by the Intramural Program of the National Institute of Child Health and Human Development and by the Lookout Foundation.

References

Adamo AM, Pasquini LA, Moreno MB, Oteiza PI, Soto EF, Pasquini JM: Effect of oxidant systems on the ubiquitylation of proteins in the central nervous system. J Neurosci Res 1999; 55:523–531.

Agar JN, Yuvaniyama P, Jack RF, Cash VL, Smith AD, Dean DR, Johnson MK: Modular organization and identification of a mononuclear iron-binding site within the NifU protein. J Biol Inorg Chem 2000;5:167–177.

Aksenov MY, Aksenova MV, Butterfield DA, Geddes JW, Markesbery WR: Protein oxidation in the brain in Alzheimer's disease. Neuroscience 2001;103:373–383.

Bence NF, Sampat RM, Kopito RR: Impairment of the ubiquitin-proteasome system by protein aggregation. Science 2001;292:1552–1555.

Bizzi A, Schaetzle B, Patton A, Gambetti P, Autilio-Gambetti L: Axonal transport of two major components of the ubiquitin system: Free ubiquitin and ubiquitin carboxyl-terminal hydrolase PGP 9.5. Brain Res 1991;548:292–299.

Brissot P, Guyader D, Loreal O, Laine F, Guillygomarc'h A, Moirand R, Deugnier Y: Clinical aspects of hemochromatosis. Transfus Sci 2000;23:193–200.

Cabiscol E, Piulats E, Echave P, Herrero E, Ros J: Oxidative stress promotes specific protein damage in Saccharomyces cerevisiae. J Biol Chem 2000;275:27393–27398.

Cassimeris L: The oncoprotein 18/stathmin family of microtubule destabilizers. Curr Opin Cell Biol 2002;14:18–24.

Chae HZ, Robison K, Poole LB, Church G, Storz G, Rhee SG: Cloning and sequencing of thiol-specific antioxidant from mammalian brain: Alkyl hydroperoxide reductase and thiol-specific antioxidant define a large family of antioxidant enzymes. Proc Natl Acad Sci USA 1994; 91:7017–7021.

Clauser KR, Hall SC, Smith DM, Webb JW, Andrews LE, Tran HM, Epstein LB, Burlingame AL: Rapid mass spectrometric peptide sequencing and mass matching for characterization of human melanoma proteins isolated by tow-dimensional PAGE. Proc Natl Acad Sci USA 1995;92:5072–5076.

Coudray C, Pucheu S, Boucher F, Arnaud J, de Leiris J, Favier A: Effect of ischemia/reperfusion sequence on cytosolic iron status and its release in the coronary effluent in isolated rat hearts. Biol Trace Elem Res 1994;41:69–75.

Dalle-Donne I, Rossi R, Giustarini D, Gagliano N, Lusini L, Milzani A, Di SP, Colombo R: Actin carbonylation: From a simple marker of protein oxidation to relevant signs of severe functional impairment. Free Radic Biol 2001;31: 1075–1083.

Davis MT, Stahl DC, Hefta SA, Lee TD: A microscale electrospray interface for on-line, capillary liquid chromatography/tandem mass spectrometry of complex peptide mixtures. Anal Chem 1995;67:4549–4556.

DeRusso PA, Philpott CC, Iwai K, Mostowski HS, Klausner RD, Rouault TA: Expression of a constitutive mutant of iron regulatory protein 1 abolishes iron homeostasis in mammalian cells. J Biol Chem 1995;270:15451–15454.

Dexter DT, Jenner P, Schapira AH, Marsden CD: Alterations in levels of iron, ferritin, and other trace metals in neurodegenerative diseases affecting the basal ganglia. Ann Neurol 1992; 32(suppl):S94–100.

Feder ME, Hofmann GE: Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. Annu Rev Physiol 1999;61:243–282.

Fenteany G, Standaert RF, Lane WS, Choi S, Corey EJ, Schreiber SL: Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. Science 1995;268:726–731.

Friguet B, Stadtman ER, Szweda LI: Modification of glucose-6-phosphate dehydrogenase by 4-hydroxy-2-nonenal. Formation of cross-linked protein that inhibits the multicatalytic protease. J Biol Chem 1994;269:21639–21643.

Galloway PG, Perry G, Gambetti P: Hirano body filaments contain actin and actin-associated proteins. J Neuropathol Exp Neurol 1987;46: 185–199.

Gerlach M, Ben-Shachar D, Riederer P, Youdim MB: Altered brain metabolism of iron as a cause of neurodegenerative diseases? J Neurochem 1994;63:793–807.

Gitlin JD: Aceruloplasminemia. Pediatr Res 1998; 44:271–276.

Grant AJ, Jessup W, Dean RT: Inefficient degradation of oxidized regions of protein molecules. Free Radic Res Commun 1993;18:259–267.

Guo B, Phillips JD, Yu Y, Leibold EA: Iron regulates the intracellular degradation of iron regulatory protein 2 by the proteasome. J Biol Chem 1995;270:21645–21651.

Hartl FU: Principles of chaperone-mediated protein folding. Philos Trans R Soc Lond [B] Biol 1995;348:107–112.

Hingorani K, Szebeni A, Olson MO: Mapping the functional domains of nucleolar protein B23. J Biol Chem 2000;275:24451–24457.

Holaska JM, Black BE, Love DC, Hanover JA, Leszyk J, Paschal BM: Calreticulin is a receptor for nuclear export. J Cell Biol 2001;152:127–140.

Honore B, Vorum H: The CREC family, a novel family of multiple EF-hand, low-affinity Ca(2+)-binding proteins localised to the secretory pathway of mammalian cells. FEBS Lett 2000;466:11–18.

Iwai K, Drake SK, Wehr NB, Weissman AM, LaVaute T, Minato N, Klausner RD, Levine RL, Rouault TA: Iron-dependent oxidation, ubiquitination, and degradation of iron regulatory protein 2: Implications for degradation of oxidized proteins. Proc Natl Acad Sci USA 1998; 95:4924–4928.

Iwai K, Klausner RD, Rouault TA: Requirements for iron-regulated degradation of the RNA binding protein, iron regulatory protein 2. EMBO J 1995;14:5350–5357.

Johnston SC, Larsen CN, Cook WJ, Wilkinson KD, Hill CP: Crystal structure of a deubiquitinating enzyme (human UCH-L3) at 1.8 Å resolution. EMBO J 1997;16:3787–3796.

Koppenol WH: The centennial of the Fenton reaction. Free Radic Biol Med 1993;15:645–651.

Kurihara LJ, Kikuchi T, Wada K, Tilghman SM: Loss of Uch-L1 and Uch-L3 leads to neurodegeneration, posterior paralysis and dysphagia. Hum Mol Genet 2001;10:1963–1970.

LaVaute T, Smith S, Cooperman S, Iwai K, Land W, Meyron-Holtz E, Drake SK, Miller G, Abu-Asab M, Tsokos M, Switzer R III, Grinberg A, Love P, Tresser N, Rouault TA: Targeted deletion of iron regulatory protein 2 causes misregulation of iron metabolism and neurodegenerative disease in mice. Nat Genet 2001;27:209–214.

Leroy E, Boyer R, Auburger G, Leube B, Ulm G, Mezey E, Harta G, Brownstein MJ, Jonnalagada S, Chernova T, Dehejia A, Lavedan C, Gasser T, Steinbach PJ, Wilkinson KD, Polymeropoulos MH: The ubiquitin pathway in Parkinson's disease. Nature 1998;395:451–452.

Levine RL, Williams JA, Stadtman ER, Shacter E: Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol 1994;233:346–357.

Levine RL, Wehr N, Williams JA, Stadtman ER, Shacter E: Determination of carbonyl groups in oxidized proteins. Methods Mol Biol 2000;99: 15–24.

Lorain S, Lecluse Y, Scamps C, Mattei MG, Lipinski M: Identification of human and mouse HIRA-interacting protein-5 (HIRIP5), two mammalian representatives in a family of phylogenetically conserved proteins with a role in the biogenesis of Fe/S proteins. Biochem Biophys Acta 2001;1517:376–383.

Lowe J, McDermott H, Landon M, Mayer RJ, Wilkinson KD: Ubiquitin carboxyl-terminal hydrolase (PGP 9.5) is selectively present in ubiquitinated inclusion bodies characteristic of human neurodegenerative diseases. J Pathol 1990;161:153–160.

Mao GD, Thomas PD, Lopaschuk GD, Poznansky MJ: Superoxide dismutase (SOD)-catalase conjugates: Role of hydrogen peroxide and the Fenton reaction in SOD toxicity. J Biol Chem 1993;268:416–420.

McCord JM: Iron, free radicals, and oxidative injury. Semin Hematol 1998;35:5–12.

Mitsumoto A, Nakagawa Y: DJ-1 is an indictor for endogenous reactive oxygen species elicited by endotoxin. Free Radic Res 2001;35:885–893.

Mitsumoto A, Nakagawa Y, Takeuchi A, Okawa K, Iwamatsu A, Takanezawa Y: Oxidized forms of peroxiredoxins and DJ-1 on two-dimensional gels increased in response to sublethal levels of paraquat. Free Radic Res 2001;35:301–310.

Mitsumoto A, Takanezawa Y, Okawa K, Iwamatsu A, Nakagawa Y: Variants of peroxiredoxins expression in response to hydroperoxide stress. Free Radic Biol Med 2001;30:625–635.

Nunez MT, Osorio A, Tapia V, Vergara A, Mura CV: Iron-induced oxidative stress up-regulates calreticulin levels in intestinal epithelial (Caco-2) cells. J Cell Biochem 2001;82:660–665.

Park BJ, Lee DG, Yu JR, Jung SK, Choi K, Lee J, Lee J, Kim YS, Lee JI, Kwon JY, Lee J, Singson A, Song WK, Eom SH, Park CS, Kim DH, Bandyopadhyay J, Ahnn J: Calreticulin, a calcium-binding molecular chaperone, is required for stress response and fertility in *Caenorhabditis elegans.* Mol Biol Cell 2001;12:2835–2845.

Perkins DN, Pappin DJ, Creasy DM, Cottrell JS: Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 1999;20:3551–3567.

Powell SR, Gurzenda EM, Wahezi SE: Actin is oxidized during myocardial ischemia. Free Radic Biol Med 2001;30:1171–1176.

Rajan RS, Illing ME, Bence NF, Kopito RR: Specificity in intracellular protein aggregation and inclusion body formation. Proc Natl Acad Sci USA 2001;98:13060–13065.

Reinheckel T, Korn S, Mohring S, Augustin W, Halangk W, Schild L: Adaptation of protein carbonyl detection to the requirements of proteome analysis demonstrated for hypoxia/reoxygenation in isolated rat liver mitochondria. Arch Biochem Biophys 2000;376:59–65.

Rhee SG, Kang SW, Netto LE, Seo MS, Stadtman ER: A family of novel peroxidases, peroxiredoxins. Biofactors 1999;10:207–209.

Rouault TA: Systemic iron metabolism: A review and implications for brain iron metabolism. Pediatr Neurol 2001;25:130–137.

Saigoh K, Wang YL, Suh JG, Yamanishi T, Sakai Y, Kiyosawa H, Harada T, Ichihara N, Wakana S, Kikuchi T, Wada K: Intragenic deletion in the gene encoding ubiquitin carboxy-terminal hydrolase in gad mice. Nat Genet 1999;23:47–51.

Sayre LM, Perry G, Smith MA: Redox metals and neurodegenerative disease. Curr Opin Chem Biol 1999;3:220–225.

Schilke B, Voisine C, Beinert H, Craig E: Evidence for a conserved system for iron metabolism in the mitochondria of *Saccharomyces cerevisiae.* Proc Natl Acad Sci USA 1999;96:10206–10211.

Schultz C, Dick EJ, Cox AB, Hubbard GB, Braak E, Braak H: Expression of stress proteins alpha B-crystallin, ubiquitin, and hsp27 in pallidonigral spheroids of aged rhesus monkeys. Neurobiol Aging 2001;22:677–682.

Sheth S, Brittenham GM: Genetic disorders affecting proteins of iron metabolism: Clinical implications. Annu Rev Med 2000;51:443–464.

Stadtman ER, Oliver CN: Metal-catalyzed oxidation of proteins. J Biol Chem 1991;266:2005–2008.

Swaiman KF: Hallervorden-Spatz syndrome. Pediatr Neurol 2001;25:102–108.

Szebeni A, Olson MO: Nucleolar protein B23 has molecular chaperone activities. Protein Sci 1999;8:905–912.

Takahashi K, Taira T, Niki T, Seino C, Iguchi-Ariga SM, Ariga H: DJ-1 positively regulates the androgen receptor by impairing the binding of PIASx alpha to the receptor. J Biol Chem 2001;276:37556–37563.

Van EJE, Powers F, Law W, Larue C, Hodges RS, Solaro RJ: Breakdown and release of myofilament proteins during ischemia and ischemia/reperfusion in rat hearts: Identification of degradation products and effects on the pCa-force relation. Circ Res 1998;82:261–271.

White BC, Sullivan JM, DeGracia DJ, O'Neil BJ, Neumar RW, Grossman LI, Rafols JA, Krause GS: Brain ischemia and reperfusion: Molecular mechanisms of neuronal injury. J Neurol Sci 2000;179:1–33.

Wilkinson KD, Laleli-Sahin E, Urbauer J, Larsen CN, Shih GH, Haas AL, Walsh ST, Wand AJ: The binding site for UCH-L3 on ubiquitin: Mutagenesis and NMR studies on the complex between ubiquitin and UCH-L3. J Mol Biol 1999;291:1067–1077.

Wilkinson KD, Lee KM, Deshpande S, Duerksen-Hughes P, Boss JM, Pohl J: The neuron-specific protein PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase. Science 1989;246:670–673.

Yates JR Jr, Eng JK, McCormack AL, Schieltz D: Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. Anal Chem 1995;67:1426–1436.

Yim MB, Chock PB, Stadtman ER: Copper, zinc superoxide dismutase catalyzes hydroxyl radical production from hydrogen peroxide. Proc Natl Acad Sci USA 1990;87:5006–5010.

Zugaro LM, Reid GE, Ji H, Eddes JS, Murphy AC, Burgess AW, Simpson RJ: Characterization of rat brain stathmin isoforms by two-dimensional gel electrophoresis-matrix assisted laser desorption/ionization and electrospray ionization-ion trap mass spectrometry. Electrophoresis 1998;19:867–876.